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Review

Structure and properties of pyruvate decarboxylase and site-directed mutagenesis of the *Zymomonas mobilis* enzyme

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Abstract

Pyruvate decarboxylase (EC 4.1.1.1) is a thiamin diphosphate-dependent enzyme that catalyzes the penultimate step in alcohol fermentation. The enzyme is widely distributed in plants and fungi but is rare in prokaryotes and absent in animals. Here we review its structure and properties with particular emphasis on how site-directed mutagenesis of the enzyme from *Zymomonas mobilis* has assisted us to understand the function of critical residues.

Keywords: Catalysis; Cofactor binding; Enzyme structure; Pyruvate decarboxylase; Thiamin diphosphate; (*Zymomonas mobilis*)

Contents

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1. Introduction

1.1. Preamble

Enzymes that use thiamin diphosphate (ThDP) as a cofactor are found in all organisms where they catalyze reactions involving the cleavage of a carbon-carbon bond that is adjacent to a keto group. The first stage in these reactions usually results in the release of the first product and formation of an enzyme-bound aldehyde. The fate of this intermediate differs between the various types of ThDP-dependent enzymes; it may be released, oxidized, or transferred to an acceptor substrate. The simplest type of reaction is where the aldehyde is released and a typical example is the non-oxidative decarboxylation of pyruvate to acetaldehyde catalyzed by pyruvate decarboxylase (PDC, EC 4.1.1.1), the penultimate step in alcohol fermentation.

$$CH_3{\cdot}CO{\cdot}COO^- + H^+ \rightarrow CH_3{\cdot}CHO + CO_2$$

In this review we shall discuss the general features of the structure and properties of PDC. While much of the published work to which we will refer concerns the yeast enzyme, some emphasis will be placed on the PDC from *Zymomonas mobilis* that has been studied in this laboratory.

1.2. Occurrence

PDC was first detected in yeast extracts in 1911 by Neuberg and Karczag [1]. The enzyme, or a corresponding DNA sequence, has since been found to be widely distributed in fungi and higher plants but it is relatively rare in prokaryotes and unknown in animals. In fungi, PDC has been found in *Saccharomyces cerevisiae*, *S. carlsbergensis* and *S. uvarum* [2–6], *Neurospora crassa* [7], *Kluyveromyces* species [8,9], *Aspergillus* species [10,11], *Hanseniaspora uvarum* [12] and in *Schizosaccharomyces pombe* [13]. PDC has been detected in a variety of plants including parsnip [14], maize [15–17], orange [18], pea and jack bean [19–21], sweet potato [22], wheat [23], cottonwood and soybean [24] and rice [25,26]. Finally, in prokaryotes, PDC has been found in *Z. mobilis* [27–30], *Sarcina ventriculi* [31,32], *Clostridium botulinum* [33], *Acetobacter* species [34] and in *Erwinia amylovora* [35]. The best studied of these prokaryotic forms, and the PDC that will be the focus of this review, is that from *Z. mobilis*. This organism is an anaerobic Gram-negative bacterium that is widely used in tropical areas for the fermentation of alcoholic beverages [36]. However, unlike fungi and plants, it converts glucose or other sugars to pyruvate via the Entner-Doudoroff pathway [37,38] and the low energy efficiency of this pathway requires the production of large quantities of ethanol. Consequently, PDC is one of the most abundant proteins present in *Z. mobilis* [39].

1.3. Subunit structure

The quaternary structure of PDC is pH-dependent; the enzyme from most sources shows maximal activity between pH 6.0 and 6.7 [12,25,32,40], the exception being PDC from oranges which has a pH optimum of 5.0 [18]. At their optimum pH the active form of PDC from yeast, *Z. mobilis*, *S. ventriculi*, and rice is tetrameric with a molecular mass of approximately 240 kDa [5,25,32,40] while PDC from maize and peas forms higher oligomers with molecular masses of up to 1 mDa [15,41,42]. At pH values near 7.5, an equilibrium exists between the dimers and tetramers of yeast PDC, but only the tetramers have been shown to be catalytically active [43]. At pH values above 8.0, all catalytic activity is lost easily due to the dissociation of the cofactors, ThDP and Mg^{2+}. Under these conditions, the yeast enzyme dissociates into dimers [43] while the more stable enzyme from *Z. mobilis* retains its tetrameric form even in the absence of cofactors [44,45]. The degree of oligomerization of PDC from peas has been shown to decrease at high pH but oligomers higher than the dimer exist [21]. From the crystal structure of yeast PDC [46–48] we now know that there is a limited number of contacts between the dimers in the tetramer. However, this loose association of the dimers presumably also exists in PDC from *Z. mobilis* which does not dissociate into dimers at elevated pH. That the dimers do not dissociate into subunits is not surprising given the number of tight contacts between the two subunits in the dimer [46].

PDC from *Z. mobilis* is a homotetramer with a measured subunit molecular weight of 59–65 kDa [29,40,49] in reasonably good agreement with the value of 60.79 kDa calculated from the DNA sequence [40]. In contrast, depending on the strain from which the enzyme is isolated and the purification method employed, the enzyme from *S. cerevisiae* is either an α_4 or β_4 homotetramer or an $\alpha_2\beta_2$ tetramer composed of two different types of subunits [5,50–53]. Two types of subunits have also been identified in PDC from wheat germ, peas, maize and rice [16,20,23,25]. Controversy still exists as to whether the smaller subunits are encoded by different structural genes or whether they are the product of proteolysis during the purification procedure. Although three structural PDC genes have been identified in yeast [4,54,55], only two of these (PDC1 and PDC5) appear to be expressed normally. It has been shown that the PDC1 gene encodes the larger subunit [56,57] but there has been no identification of a structural gene that might encode the second subunit. The smaller subunit in yeast PDC is therefore considered to be the product of proteolysis [5,58]. While this conclusion is in conflict with the measured amino acid compositions of the subunits [59] these composition data are inconsistent with that deduced from either the PDC1 and PDC5 gene sequences. As amino acid sequencing of the isolated smaller subunit has not been performed, the origin of the second subunit remains as an open question. Multiple PDC genes have also been identified in plants where most are induced (at the mRNA level) by hypoxic stress [21,26,60].

The most unusual subunit structure possessed by any PDC is that from *N. crassa*. This organism contains large filament bundles composed of a 59-kDa subunit [61] that was later shown to be PDC [7]. Curiously, phylogenetic analysis of the protein sequence has shown that this *N. crassa* PDC is more closely related to *Z. mobilis* PDC than to any of the fungal enzymes ([62] and Duggleby, unpublished).

1.4. Cofactors

PDC exhibits substantial activity in the absence of added cofactors and it is only by treatment above pH 8 to remove bound ThDP and Mg^{2+} that the absolute requirement for both can be demonstrated [44]. The loss of catalytic activity and dissociation of cofactors above pH 8 is reversible and, at pH values near 6.0, the holoenzyme is reconstituted and activity restored to almost 100%. Diefenbach and Duggleby [44] have proposed a model for the reactivation of the *Z. mobilis* PDC apoenzyme by its cofactors. Similar to that proposed by Schellenberger and Hübner [63] for yeast PDC, this model allows for the independent and reversible binding of either ThDP or Mg^{2+} to the apoenzyme. This is followed by a slow quasi-irreversible binding of the second cofactor to form the catalytically active holoenzyme. The binding is highly cooperative in that the presence of one cofactor greatly increases the affinity for the second. Circular dichroism and X-ray solution scattering

studies have shown this reconstitution of the active holoenzyme to involve conformational changes of the dimers [43,50,64].

Considerable activity is observed when Mg^{2+} is replaced by other divalent metal ions such as Mn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, Cd^{2+} and Zn^{2+} [42,65,66]. Studies on the effect of ThDP derivatives and diphosphate esters of thiazolium compounds on reconstitution of yeast PDC have shown that both the pyrimidine ring and the diphosphate group are required for the formation of the active tetramer [67,68]. The role of various parts of the pyrimidine ring have been investigated by reconstitution of yeast PDC with several analogs [69–72]. The N-1′ atom is essential for binding while the 4′-amino is not required for binding but is required for catalysis.

1.5. Kinetic properties

Z. mobilis PDC exhibits normal Michaelis-Menten kinetics (Fig. 1) with a K_m for pyruvate ranging from 0.3 to 4.4 mM [28,29,40,44,73]. In this respect it appears to be unusual as the remaining PDCs that have been characterized to date exhibit sigmoidal kinetics [16,21,32,74,75], and it has been concluded that the substrate also functions as an essential activator of the enzyme [76]. The half-saturating substrate concentration ($S_{0.5}$) for pyruvate of yeast PDC is in the range of 1–3 mM [77] with a Hill coefficient between 1.4 and 1.9 [74,75,78], while the $S_{0.5}$ for other PDCs range from 0.25 mM in rice [25] to 13 mM in *S. ventriculi* [32] with Hill coefficients of around 2.0.

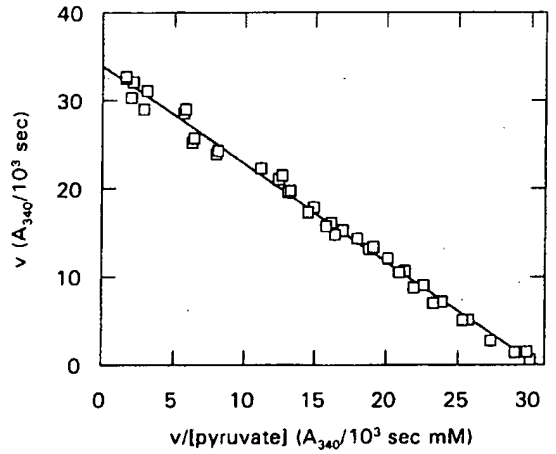

Fig. 1. Kinetics of *Z. mobilis* PDC with respect to pyruvate concentration. The rate of the enzyme-catalyzed reaction was measured as a function of the pyruvate concentration, over the range $0.029 \times K_m$ to $18 \times K_m$ (3–95% of V_m). The linearity of the Eadie-Hofstee plot demonstrates that the data conform to a hyperbolic saturation curve over the entire range. Data were taken from Diefenbach and Duggleby [44].

Pyruvamide can substitute for pyruvate in activation and the addition of this analog transforms the shape of the rate vs. [pyruvate] plot into a hyperbolic one for yeast [57,76] and wheat germ [23] PDC. It was proposed from X-ray scattering studies that pyruvamide induces structural changes in the dimers which result in less compact tetramers [79]. In the remainder of this review, to facilitate comparison between *Z. mobilis* and yeast PDC, residue numbers will be prefixed with 'z' or 'y', respectively. The activation of yeast PDC by pyruvate or analogs has been shown to involve yC221; mutagenesis to serine abolishes the activation [80]. It appears that activation involves formation of a hemiketal which perturbs the subunit interface and Sun et al. [81,82] have proposed that this opens the active site allowing access by the substrate. Recently the structure of yeast PDC crystallized in the presence of pyruvamide has been determined [48]. In contrast to the suggestion of König et al. [79], it has a more compact structure. Moreover, the active site appears to be no more accessible than when pyruvamide is absent so the precise molecular mechanism of activation remains uncertain. Nevertheless, activation seems to involve a conformational change as the enzyme can be locked into its activated form by cross-linking with bifunctional imidoesters [83].

Although the evidence implicating yC221 in substrate activation of yeast PDC seems clear, a sigmoidal pyruvate saturation curve is also observed for PDC from plants but the equivalent amino acid in plant PDC sequences is lysine [21], as it is for *Z. mobilis* PDC. Indeed, there is no cysteine conserved between yeast and plant PDCs. This suggests that substrate activation of plant PDC is mediated through a different mechanism, perhaps involving reaction with a different cysteine but leading to a similar conformational change.

Yeast PDC is able to decarboxylate a wide range of substrates in which the methyl group of pyruvate is replaced with a larger alkyl, or an aryl, substituent [84]. While glyoxylate is also decarboxylated, the resultant formaldehyde is not released causing progres-

sive and irreversible enzyme inactivation [85]. It has been shown that 3-fluoropyruvate is a substrate for both yeast [82] and wheat-germ [86] PDC but in this case decarboxylation is followed by release of fluoride ion and the final product is acetate. A similar reaction (but releasing hydroxide ion) has been suggested for *Z. mobilis* PDC acting on 3-hydroxypyruvate; however, for this enzyme the liberation of acetate is slow and 3-hydroxypyruvate behaves as a progressive, but slowly reversible, inhibitor [87]. In contrast to the yeast enzyme, *Z. mobilis* PDC acts on only a restricted range of substrates (Huang and

Table 1
Alignment of selected regions of pyruvate decarboxylases

	A ‡	B ‡ ‡‡	C ‡ ‡ ‡ ‡ ‡
Zmo (X59558)	EQVYCCNELNCGFSAE	GAPNNNDHAAGHVLHHALGKTD	RNILMVGDGSFQLTAQEVAQMVRLKLPVIIFLINNYGYTIEVMIH--DGPYNNIKNWDYAGLMEVFNG
Sce1 (X77316)	RWAGNANELNARYAAD	GVPSISSQAKQLLLHHTLGNGD	RVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLIHGPKAQYNEIQGWDHLSLLPTFGA
Sce5 (X15668)	RWAGNANELNAAYAAD	GVPSISSQAKQLLLHHTLGNGD	RVILFIGDGSLQLTVQEISTMIRWGLKPYIFVLNNNGYTIEKLIHGPHAEYNEIQGWDHLALLPTFGA
Sce6 (X55905)	RWAGNANELNAAYAAD	GVPSISAQAKQLLLHHTLGNGD	RVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLIHGPHAEYNEIQTWDHLALLPAFGA
Spo (Z81312)	SEIGCCNELNCAFAAE	GSPNTNDLSSGHLLHHTLGTHD	RTIVMVGDGSFQLTGQEISQMIRHKLPVLIFLLNNRGYTIEIQIH--DGPYNRIQNWDFAAFCESLNG
Huv (U13635)	RWAASLNELNAAYAAD	GVPSLASQAKQLLLHHTLGNGD	RVILFVGDGSLQLTVQEIACLIRWGLKPYIFVLNNNGYTIEKLIHGPTAQYNMIQNWKQLRYLTNFGA
Kla (X85968)	RWAGNANELNAAYAAD	GVPSVSSQAKQLLLHHTLGNGD	RVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIERLIHGETAQYNCIQNWQHLELLPTFGA
Kma (L09727)	RWAGNANELNAAYAAD	GVPSISSQAKQLLLHHTLGNGD	RVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIERLIHGETAQYNCIQSWKHLDLLPTFGA
Ncr (U65927)	KEVGCANELNCSLAAE	GSPNTNDPSQYHILHHTLGHPD	HTIVLVGDGSFQVTAQEVSQMVRFKVPITIMLINNRGYTIEVEIH--DGSYNKIKNWDYAMLVEAFNS
Apa (U00967)	KWVGNCNELNAGYAAD	GTPMRASQESRALIHHTFNDGD	RTILFIGDGSFQMTVQELSTIIHQKLNVIIFLINNDGYTIERCIHGRNQAYNDVAPWRYLKAAEFFGA
Nta1 (X81854)	partial sequence	GGPNSNDYGTNRILHHTIGLQD	RVIACIGDGSFQVTAQDISTMLRCGQRTIIFLINNGGYT partial sequence
Nta2 (X81855)	KNIGCCNELNAGYAAD	GGPNTNDYGTNRILHHTIGLPD	RVISCIGDGSFQVTAQDVSTMIRCEQKNIIFLINNGGYTIEVEIH--DGPYNVIKNWNYTGLVDAIHN
Psa1 (Z66543)	NLVGCCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVIACIGDGSFQVTAQDISTMIRCGQRSIIFLINNGGYTIEVEIH--DGPYNVIKNWDYTGFVSAIHN
Ath1 (U71121)	NLIGCCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVLAFIGDGSFQVTVQDISTMLRNGQKTIIFLINNGGYTIEVEIH--DGPYNVIKNWNYTGLVDAIHN
Ath2 (U71122)	KLIGCCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVIACIGDGSFQVTAQDVSPMIRCGHKTIIFLINNGGYTIEVEIH--DGPYNVIKNWNYTAFVEAIHN
Zma1 (X59546)	TLVGCCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVIACIGDGSFQVTAQDVSTMLRCGQKSIIFLINNGGYTIEVEIH--DGPYNVIKNWDYTGLVNAIHN
Osa1 (U26660)	KLIACCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVISCIGDGSFQMTAQDVSTMLRCGQKSIIFLINNGGYTIEVEIH--DGPYNVIKNWDYTGLIDAIHN
Osa2 (U27350)	LLVGCCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVIACIGDGSFQVTAQDVSTMIRCAQNSIIFLINNGGYTIEVEIH--DGPYNVIKNWNYTGLVDAIHN
Osa3 (U07338)	SVVGCCNELNAGYAAD	GGPNSNDYGTNRILHHTIGLPD	RVVACIGDGSFQVTAQDVSTMLRCGQKSIIFLINNGGYTIEVEIH--DGPYNVIKNWDYVGLVNAIHN

(A) The sequences surrounding the catalytic glutamate (E50 in *Z. mobilis* PDC, see Section 4). (B) The region near the mobile loop (underlined, see Section 5) and the pair of invariant histidines (H113 and H114, see Section 4). (C) The region encompassing the ThDP motif (underlined, see Section 2): residues 439–467. Residues marked ‡ indicate the positions of *Z. mobilis* PDC mutants that are discussed in the text. Species abbreviations are: Apa, *Aspergillus parasiticus*; Ath, *Arabidopsis thaliana*; Huv, *Hanseniaspora uvarum*; Kla, *Kluyveromyces lactis*; Kma, *Kluyveromyces marxianus*; Ncr, *Neurospora crassa*; Nta, *Nicotiana tabacum*; Osa, *Oryza sativa*; Psa, *Pisum sativum*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*; Zma, *Zea mays*; and Zmo, *Zymomonas mobilis*. Sequences were aligned using ClustalW [98] then manually adjusted to remove any anomalies.

Duggleby, unpublished): it will accept 2-ketobutyrate quite well, but shows low activity on 2-ketovalerate and no detectable activity towards 2-ketoisovalerate.

Phosphate has been shown to enhance cooperativity in PDC from various yeasts (*S. carlsbergensis* [88], *S. cerevisiae* and *C. utilis* [89]) while simultaneously reducing the affinity for pyruvate. We have shown [90] a decrease in affinity for substrate in *S. cerevisiae* PDC (21-fold over the range 0–400 mM phosphate) which is similar to that shown for PDC from *S. carlsbergensis* and *C. utilis*, but which is lower than the values reported by van Urk et al. [89] for *S. cerevisiae* PDC. Studies of X-ray scattering have shown that phosphate stabilizes the tetramer by shifting the dimer-tetramer equilibrium to higher pH values, without altering the conformation of the tetramers [79]. This enhanced interaction in the tetramers is the suggested explanation of how phosphate increases cooperativity. The inhibition of PDC by phosphate is thought to play a role, along with substrate activation, in the regulation of fermentation in yeast. We have observed that PDC from *Z. mobilis* also displays a decreased affinity for pyruvate in the presence of phosphate. However, in contrast to yeast PDC, it shows Michaelis-Menten kinetics over a 0–200 mM phosphate range [90]. It is not known whether the inhibition by phosphate has any physiological implications within the *Z. mobilis* cells.

2. Cofactor binding

2.1. The ThDP motif

Although the basic mechanism of non-enzymatic, thiamin-catalyzed reactions is fairly well understood [66,91,92], our comprehension of catalysis in ThDP-dependent enzymes has, until fairly recently, been limited by the absence of a three-dimensional structure for any of these enzymes. Information on the cofactor binding site and the identification of certain amino acid residue types postulated to be involved in catalysis have come from tryptophan fluorescence, cysteine reactivity, X-ray scattering and circular dichroism studies [77,78,93–96]. The determination of the amino acid sequences of several of these enzymes and their comparison, led to the identification of a common structural motif shared by all these enzymes [97]. As this motif ($GDGX_{24-27}NN$) is the only sequence shared by these ThDP-dependent enzymes, it was proposed that the motif may be involved in cofactor binding. This ThDP-binding motif has since been found in a large number of PDCs (Table 1C) and other ThDP-dependent enzymes (Table 2).

2.2. The crystal structure

The determination of the crystal structures of three ThDP-dependent enzymes (transketolase by Lindqvist et al. [99]; pyruvate oxidase by Muller and Schulz [100]; and yeast PDC by Dyda et al. [46]) has confirmed the role of this motif in the binding of ThDP, and has enabled us to better understand the role of the critical residues. Despite the differences in quaternary structure, reactions catalyzed, and amino acid sequence (less than 16% homology), the folding pattern and the conformation of the ThDP binding region is strongly conserved in all three enzymes. Each subunit is composed of three domains of approximately equal size. In PDC and pyruvate oxidase, the first domain (α) binds the pyrimidine ring of ThDP while the last domain (γ), which contains the ThDP motif, interacts with Mg^{2+} and the diphosphate group of ThDP. In transketolase, the positions of the domains are different with the metal ion and diphosphate binding site at the amino-terminus and the pyrimidine binding site in the middle domain. In all cases, the ThDP and metal ion are situated at the subunit interface. The ThDP-binding region of PDC is illustrated in Fig. 2.

2.3. Site-directed mutagenesis in the ThDP motif

In order to better understand the function of residues within the motif, we have mutated some of the conserved residues. We chose to perform these studies on *Z. mobilis* PDC for several reasons. First, the gene had been isolated and cloned, and the protein expressed in *Escherichia coli* [30], all of which are important requirements for site-directed mutagenesis. Second, the enzyme is easily purified, stable and shows simple kinetics towards pyruvate making analysis of the properties of mutants relatively straightforward. Third, tryptophan fluorescence in the

Table 2
ThDP-binding motif of selected enzymes

A. Acetohydroxyacid synthases

EcoI	(X02541)	GDGSLMMNIQEMATASENQLDVKIILMNN
EcoII	(M10313)	GDGSFMMNVQELGTVKRKQLPLKIVLLDN
EcoIII	(X01609)	GDGSIQMNIQELSTALQYELPVLVVNLNN
Bfl	(D13290)	GDGCFQMTNQELTTAAVEGFPIKIALINN
Ccr	(L25317)	GEASIQMCIQELSTAIQFDLPVKIFILNN
Cgl	(L09232)	GDGCFQMTNQELTTAAVEGFPIKIALINN
Bsu1	(L03181)	GDGGFQMTLQELDVIRELNLPVKVVILNN
Bsu2	(L04470)	GDGGFLFSAMELETAVRLKAPIVHIVWND
Kte	(L04507)	GDGGFLQSSMELETAVRLHANILHIIWVD
Kpn	(M73842)	GDGGFLQSSMELETAVRLKANVLHLIWVD
Lla	(L16975)	GDGGFLFTGQELETAVRLNLPIVQIIWND
SplX	(M75906)	GDASVQMNIQELGTIAQYGINVKTVIINN
SplY	(M75907)	GDGSFQMNMQELGTIAQYGIGVKVIILNN
Asp	(U55852)	GDGSFIMNVQELATIRVENLPVKIMLLNN
Sce	(X02549)	GDASFNMTLTELSSAVQAGTPVKILILNN
Spo	(L11293)	GDASFSMTGMELATVRQFDIPVKILILNN
Pum	(M94625)	GDSSFQMNMQELGTIAQYKLPIKIVIINN
Ath	(X51514)	GDGSFIMNVQELATIRVENLPVKVLLLNN
NtaA	(X07644)	GDGSFIMNVQELATIKVENLPVKIMLLNN
NtaB	(X07645)	GDGSFIMNVQELATIKVENLPVKIMLLNN
BnaI	(Z11524)	GDGSFIMNVQELATIRVENLPVKILLLNN
BnaII	(Z11525)	GDGSFIMNIQELATIRVENLPVKVLLINN
BnaIII	(Z11526)	GDGSFIMNVQELATIRVENLPVKILLLNN
Zma108	(X63553)	GDGSFLMNVQELAMIRIENLPVKVFVLNN
Zma109	(X63554)	GDGSFLMNIQELAMIRIENLPVKVFVLNN

B. E1 component of 2-ketoacid and acetoin dehydrogenase

MtuPDH	(Q10504)	GDGEMD-EPESRGLAHVGALEGLDN--LTFVINCN
MgePDH	(P47516)	GDGGTA-EGEFYEAMNIASIHKWN---TVFCINNN
BstPDH	(P21873)	GDGGTS-QGDFYEGINFAGAFKAP---AIFVVQNN
BsuPDH	(P21881)	GDGGRS-QGDFYEGINFAGAYKAP---AIFVVQNN
AlaPDH	(P35485)	GDGGTA-HGEFYEGLNFAASFKAP---VVAVIQNN
SpoPDH	(Q10489)	GDGASN-QGQAFEAFNMAKLWGLP---VIFACENN
ScePDH	(P16387)	GDGASN-QGQVFESFNMAKLWNLP---VVFCCENN
PpurPDH	(P51267)	GDGTTN-NGQFFECLNMAVLWKLP---IIFVVENN
AthPDH	(P52901)	GDGAAN-QGQLFEALNISALWDLP---AILVCENN
StuPDH	(P52903)	GDGAAN-QGQLFEALNMAALWDLP---AILVCENN
PsaPDH	(P52902)	GDGAAN-QGQLFEALNISALWDLP---AILVCENN
AsuPDH1	(P26267)	GDGATN-QGQLFESMNMAKLWDLP---VLYVCENN
AsuPDH2	(P26268)	GDGATN-QGQLYESMNMAKLWELP---VLYVCENN
CelPDH	(P52899)	GDGAAN-QGQLFEATNMAKLWDLP---VLFVCENN
SscPDH	(P29804)	GDGAAN-QGQIFEAYNMAALWKLP---CVFICENN
MmuPDH1	(P35486)	GDGAAN-QGQIFEAYNMAALWKLP---CIFICENN
MmuPDH2	(P35487)	GDGAAN-QGQVFEAYNMSALWKLP---CVFICENN
RnoPDH1	(P26284)	GDGAAN-QGQIFEAYNMAALWKLP---CIFICENN
RnoPDH2	(Q06437)	GDGAAN-QGQVFEAYNMSALWKLP---CVFICENN
SmaPDH	(P52900)	GDGAAN-QGQIFEAYNMAALWKLP---CIFICENN
HsaPDH1	(P08559)	GDGAAN-QGQIFEAYNMAALWKLP---CIFICENN
HsaPDH2	(P29803)	GDGAAN-QGQIAEAFNMAALWKLP---CVFICENN
EcoKGDH	(P07015)	GDAAVTGQGVVQETLNMSKARGYEVGGTVRIVINN
HinKGDH	(P45303)	GDSAVAGQGVVQETLNMSNTRGYSVGGTIRIVINN
HsaKGDH	(Q02218)	GDAAFAGQGIVYETFHLSDLPSYTTHGTVHVVVNN
PputBCDH	(M57613)	GDGATA-ESDFHTALTFAHVYRAP---VILNVVNN
MmuBCDH	(L47335)	GEGAAS-EGDAHAGFNFAATLECP---IIFFCRNN
RnoBCDH	(J02827)	GEGAAS-EGDAHAGFNFAATLECP---IIFFCRNN
HsaBCDH	(J04474)	GEGAAS-EGDAHAGFNFAATLECP---IIFFCRNN
BsuACDH	(AF006075)	GDGANN-QGTFHEGLNLAAVWNLP---VVFVAENN
PputACDH	(L35343)	GDGASN-EGAVFEAMNMASIMNLP---CIFVAENN
PcaACDH	(U01100)	GDGASN-QGTNFESMNFAVTLDLP---MIFVLENN
CmaACDH	(L31844)	GDASTN-QGTFHEALNMASVWKLP---IVFVCENN
AeuACDH	(M66060)	GDGASN-QGTFLESLNLAAVWNLP---VIFVIENN
KpnACDH	(U00985)	GDGGSN-QGLVFEAINMAVVLQLP---AVFIFENN

C. Transketolases

Eco1	(X68025)	GDGCMMEGISHEVCSLAGTLKLGKLIAFYDDN
Eco2	(D12473)	GDGCLMEGISHEVCSLAGTLGLGKLIGFYDHN
Rsp	(M68914)	GDGCLMEGISHEAIDMGGHLGLGRLIVLWDDN
Rca	(L48803)	GDGCLMEGISQEAIGLAGKQELDNLIVLWDNN
Xfl	(U29134)	GDGCLMEGVSHEACSLAGRLGLGKLVAFYDDN
Aeu	(M68905)	GDGCLMEGLSHEACSLAGTLGLGKLICLYDDN
Hin	(U32783)	GDGCLMEGISHEACSLAGTLGLGKLIAFYDDN
Mle	(U00013)	SDGDIEEGVTSEASSLAAVQQLGNLIVFYDHN
Mge	(U39686)	GDGDLQEGVSYEVSQIAGLYKLNKLIVLHDSN
Spn	(M31296)	GDGDLMEGVSSEAASYAGXXKLDKLVVLYDSN
Sce1	(X73224)	GDGCLQEGISSEASSLAGHLKLGNLIAIYDDN
Sce2	(X73532)	GDGCLQEGVSSETSSLAGHLQLGNLITFYDSN
Pst	(Z26486)	GDGCLMEGVSSEASSLAGHLQLGNLIAFWDDN
Hpo	(X02424)	GDACLQEGPALESISLAGHMGLDNLIVLYDNN
Cpl3	(Z46646)	GDGCQMEGVSNEACSIAAHWGLGKLIALYDDN
Cpl7	(Z46648)	GDGCQMEGVSNEACSLAAHWGLGKLIALYDDN
Cpl10	(Z46647)	GDGCQMEGISNEASSLAAHWGLGKLIALYDDN
Stu	(Z50099)	GDGCQMEGISNEVCSLAGHWGLGKLIAFYDDN
Mmu	(U05809)	GDGEVSEGSVWEAMAFAGIYKLDNLVAIFDIN
Rno	(U09256)	GDGEVSEGSVWEAMAFAGIYKLDNLVAIFDIN
Hsa	(U55017)	GDGELSEGSVWEAMAFASIYKLDNLVAILDIN

D. Other

EclIPDC	(D90214)	GDGAAQLTIQE-LGSMLRDKQHPIILVLNN
AbrIPDC	(L26240)	GDGAFQMTGWE-LGNCRRLGIDPIVILFNN
EcoKGDC	(L04464)	GDLSALYDLNA-LALLRQVSAPLVLIVVNN
PputBFDC	(P20906)	GDGSANYSISA-LWTAAQYNIPTIFVIMNN
OfoOCDC	(M77128)	GDSAFGFSGME-LETICRYNLPVTVIIMNN
EcoPOX	(X04105)	GDGGFSMLMGD-FLSVVQMKLPVKIVVFNN
HhaPFO	(X64521)	GDGDGYGIGGNHFMHTARENHDITYIVFNN
SsoPFO	(P72579)	GDGDLLGIGAGHFVAAGRRNVDMVVILHDN
MjaPFO	(Q57957)	GDGDLAAIGGNHFIHGCRRNIDLTVICINN
EcoGCL	(L03845)	GDFDFQFLIEE-LAVGAQFNIPYIHVLVNN
PflBZL	(U04048)	GDGSVGYSIGE-FDTLVRKQLPLIVIIMNN

(A) Acetohydroxyacid synthases and acetolactate synthases. (B) E1 component of 2-ketoacid and acetoin dehydrogenase multienzyme complexes. Individual enzymes are indicated by PDH (pyruvate dehydrogenase), KGDH (2-ketoglutarate dehydrogenase), BCDH (branched-chain 2-ketoacid dehydrogenase) and ACDH (acetoin dehydrogenase). The alignment for KGDH differs slightly from that of Hawkins et al. [97] who placed the start of the motif at the GQG sequence that is six residues C-terminal to the start that we used. (C) Transketolases. (D) Other ThDP-dependent enzymes, indicated by IPDC (indolepyruvate decarboxylase), KGDC (2-ketoglutarate decarboxylase), BFDC (benzoylformate decarboxylase), OCDC (oxalyl-CoA decarboxylase), POX (pyruvate oxidase), PFO (pyruvate:ferredoxin oxidoreductase), GCL (glyoxylate carboligase) and BZL (benzaldehyde lyase). Species abbreviations are as for Table 1, plus: Abr, *Azospirillum brasilense*; Aeu, *Alcaligenes eutrophus*; Ala, *Acholeplasma laidlawii*; Asp, *Amaranthus* sp.; Asu, *Ascaris suum*; Bfl, *Brevibacterium flavum*; Bna, *Brassica napus*; Bst, *Bacillus stearothermophilus*; Bsu, *Bacillus subtilis*; Ccr, *Caulobacter crescentus*; Cel, *Caenorhabditis elegans*; Cgl, *Corynebacterium glutamicum*; Cma, *Clostridium magnum*; Cpl, *Craterostigma plantagineum*; Ecl, *Enterobacter cloacae*; Eco, *Escherichia coli*; Hsa, *Homo sapiens*; Hha, *Halobacterium halobium*; Hin, *Haemophilus influenzae*; Hpo, *Hansenula polymorpha*; Kpn, *Klebsiella pneumoniae*; Kte, *Klebsiella terrigena*; Lla, *Lactococcus lactis*; Mge, *Mycoplasma genitalium*; Mja, *Methanococcus jannaschii*; Mle, *Mycobacterium leprae*; Mmu, *Mus musculus*; Mtu, *Mycobacterium tuberculosis*; Ofo, *Oxalobacter formigenes*; Pca, *Pelobacter carbinolicus*; Pfl, *Pseudomonas fluorescens*; Ppur, *Porphyra purpurea*; Pput, *Pseudomonas putida*; Pst, *Pichia stipitus*; Pum, *Porphyra umbilicus*; Rca, *Rhodobacter capsulatas*; Rno, *Rattus norvegicus*; Rsp, *Rhodobacter spheroides*; Sma, *Sminthopsis macroura*; Spl, *Spirulina platensis*; Spn, *Streptococcus pneumoniae*; Ssc, *Sus scrofa*; Sso, *Sulfolobus solfataricus*; Stu, *Solanum tuberosum*; and Xfl, *Xanthobacter flavus*. Sequences were aligned using ClustalW [98] then manually adjusted to remove any anomalies.

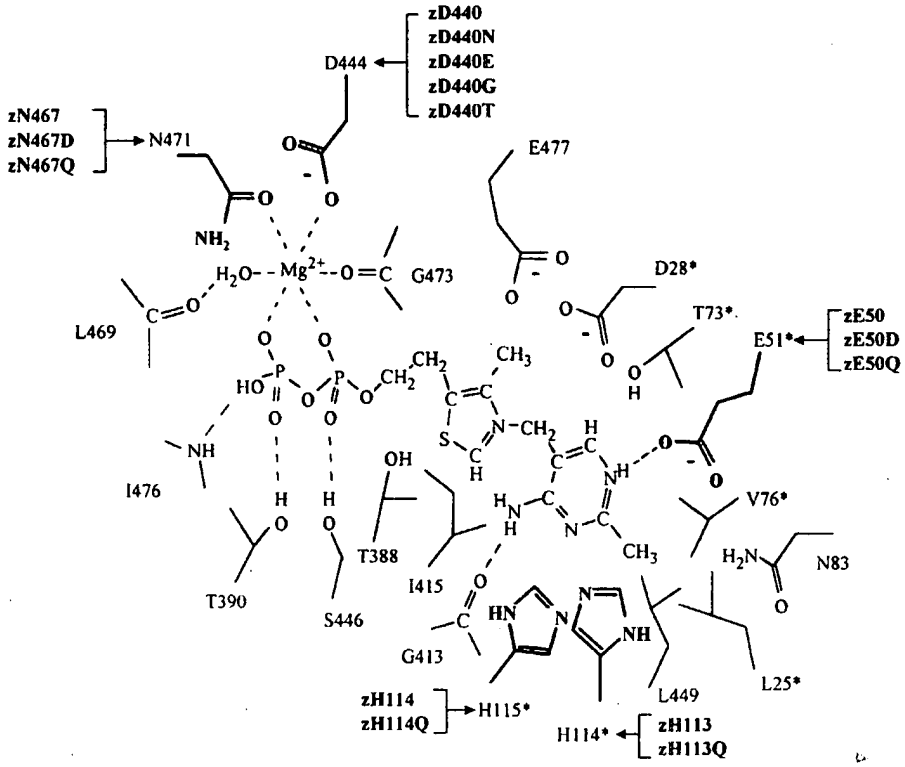

Fig. 2. Cofactor-binding site of PDC. Schematic representation of the residues that are close to the bound Mg^{2+} and ThDP of yeast PDC, adapted from Dyda et al. [46]. Residues marked with and without asterisks are located in different subunits. The side-chains of amino acids that we have mutated in *Z. mobilis* PDC are shown darker, with the corresponding residue and the mutants studied listed alongside the yeast PDC residue.

apoenzyme of *Z. mobilis* PDC is partially quenched upon adding Mg^{2+} plus ThDP (Fig. 3). By exploiting this tryptophan fluorescence quenching by cofactors, we are able to measure cofactor binding even for those mutants that are totally inactive. We initially thought that this quenching indicated the presence of a tryptophan residue within the cofactor-binding site [44] but subsequent identification of the residue concerned, coupled with determination of the crystal structure of yeast PDC [46], showed that this tryptophan is at the subunit interface. The kinetic properties with respect to pyruvate and cofactors for the mutants to be discussed below are summarized in Table 3.

We started with the aspartate in the GDG triplet, zD440 (yD444); substitution of this aspartate, which is conserved amongst virtually all ThDP-dependent enzymes, with glycine, threonine and asparagine yielded inactive proteins. These do not contain any ThDP when isolated and they are unable to bind this cofactor (Table 3) when it is added at high concentration [101]. The absence of ThDP binding by these mutants verified the role of this structural motif in ThDP binding and suggested the importance of a negative charge at this position. A glutamate at the corresponding position in a few ThDP-dependent enzymes (Table 2) led us to investigate this substitution in PDC [73]. The zD440E mutant is active but, unlike the wild-type enzyme, exhibits a lag phase in product formation which can be reduced by preincubation with extremely high concentrations (5 mM) of ThDP. The zD440E holoenzyme and apoenzyme exist in a dimeric form in contrast to wild-type where both are tetrameric [44]. Therefore, the lag phase in product formation may represent the time required for the reassociation of the dimers into the catalytically active tetramers The half-saturating concentration for ThDP of 1.49 mM for zD440E apoenzyme represents a 600-fold decrease in affinity for this cofactor while the affinity for Mg^{2+} shows a 60-fold

reduction. In contrast, the K_m for pyruvate is close to normal (Table 3).

Replacement of zN467 (yN471), the second asparagine of the doublet at the end of this ThDP motif, with aspartate yields an active enzyme while substitution with glutamine results in an inactive mutant that is unable to bind the cofactors [73]. Although the affinity of the zN467D mutant for Mg^{2+} is similar to that of the wild-type PDC, its affinity for ThDP is markedly decreased (Table 3). This decrease in ThDP affinity is of the same magnitude as that displayed by zD440E but the prominent lag phase in product formation is not evident.

From the crystal structure of PDC (Fig. 2) we now know that neither zD440 (yD444) nor zN467 (yN471) bind the ThDP directly [46]. Interestingly, none of the residues within the ThDP motif bind the ThDP. However, the Mg^{2+} is bound to the side chain oxygens of zD440 and zN467, to the backbone oxygen of zG469 (yG473), to two oxygens of the diphosphate, and to the backbone oxygen of zL465 (yL469) via a water molecule. This direct bond between the carboxyl of zD440 and the Mg^{2+} may be why a negatively charged residue is required at this position. While zD440E retains the negative charge, the longer side-chain must force the carboxyl group further into the cofactor-binding region. This would make it more difficult for the cofactors to bind, resulting in the large decreases in cofactor affinity that we have observed. We do not understand why this substitution should cause the enzyme to be dimeric given that wild-type PDC from *Z. mobilis* exists as a tetramer even in the absence of cofactors and that zD440 is not directly involved in making contacts between the dimers in the tetramer.

At the other end of the motif, replacement of zN467 with glutamine results in an inactive mutant whereas replacement with aspartate gives an active enzyme. This suggests that the size of the residue is more important than the charge. Substitution with glutamine would result in the carboxamido group being forced further into the cofactor-binding region where it will interfere with the binding of the Mg^{2+}.

Although we have not investigated zN466 (yN470) or the conserved glycines that lie either side of zD440 in PDC, Russell and coworkers [102] have mutated the second glycine (zG441) in the pyruvate dehydro-

Table 3
Kinetic properties of wild-type and mutant *Z. mobilis* PDC with respect to substrate and cofactors

Enzyme	K_m for pyruvate (mM)	$S_{0.5}$ for ThDP (μM)	$S_{0.5}$ for Mg^{2+} (μM)
Wild-type	0.52 ± 0.05	2.53 ± 0.16	5.87 ± 0.43
E50D	0.50 ± 0.02	113 ± 5	84.1 ± 4.2
E50Q[a]	ND	10.7 ± 4.0	194 ± 65
V111A	1.33 ± 0.10	1.26 ± 0.15	4.51 ± 0.98
H113Q[a]	[b]	75 ± 9	105 ± 15
H114Q	0.71 ± 0.04	1.25 ± 0.12	14.9 ± 2.8
D440N	[b]	[c]	[c]
D440T	[b]	[c]	[c]
D440G	[b]	[c]	[c]
D440E	0.95 ± 0.03	1485 ± 365	346 ± 58
E449D	0.52 ± 0.04	2.11 ± 0.16	7.26 ± 0.31
N467Q	[b]	[c]	[c]
N467D	0.95 ± 0.02	1466 ± 141	4.84 ± 0.76
W487L	0.86 ± 0.05	9.0 ± 1.7	45.0 ± 7.3
F496L	0.97 ± 0.02	0.92 ± 0.03	7.30 ± 0.31
F496I	1.11 ± 0.06	0.87 ± 0.03	8.12 ± 0.38
F496H	1.06 ± 0.05	1.60 ± 0.06	7.47 ± 0.50

Data were compiled from Diefenbach et al. [44], Candy and Duggleby [73], Candy et al. [104], Schenk et al. [109] and Huang and Duggleby (unpublished).

[a] $S_{0.5}$ for cofactors determined from tryptophan fluorescence quenching measurements. ND, not determined.

[b] Inactive.

[c] No effect on fluorescence by added cofactor.

genase complex. Substitution with alanine, serine and methionine results in little or no activity. The low activity of the alanine mutant is surprising given that this substitution occurs naturally in several ThDP-dependent enzymes. Presumably there are differences in fine structure around this residue and it may be relevant that in the structure of yeast transketolase the equivalent residue (G158) forms a hydrogen bond to an oxygen of the diphosphate group [99] while yG445 (zG441) of PDC does not participate in interactions with the cofactors [46].

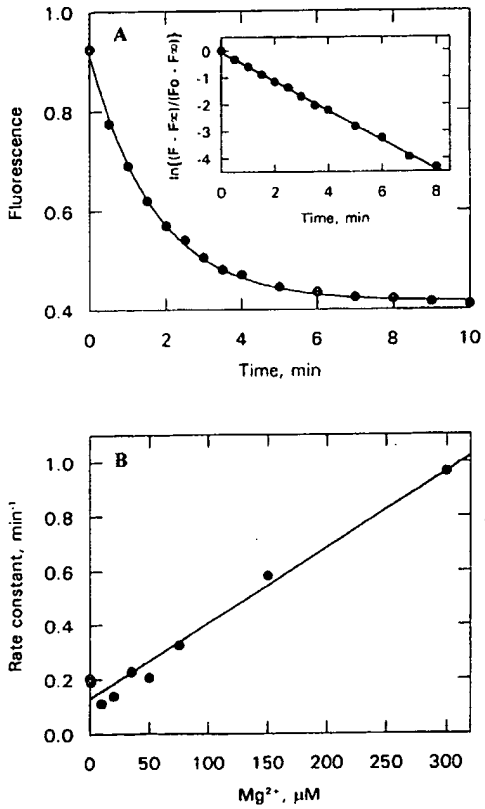

Fig. 3. Analysis of cofactor binding to *Z. mobilis* PDC by tryptophan fluorescence quenching. Apoenzyme (1.85 μM subunits) was added to a solution containing of 150 μM Mg^{2+} and 100 μM ThDP, and the decline in fluorescence (excitation at 300 nm, emission at 340 nm) followed with time as shown in A. Analysis of the data as a first-order decline from an initial (F_o) to a final (F_∞) fluorescence is shown in the inset: the negative slope of this line yields an apparent first-order rate constant. Estimation of this rate constant over a range of concentrations of Mg^{2+} (B) or ThDP (not shown) allowed determination of the apparent rate constants for binding and release, and the dissociation constant, for each cofactor. These data are adapted from Diefenbach and Duggleby [44].

The ThDP motif in PDC contains several other invariant residues (Table 1) but the role of none of these has been investigated by site-directed mutagenesis. However, zE449 (yE453), which is conserved amongst the vast majority of ThDP-dependent enzymes (Tables 1 and 2), can be replaced with aspartate in *Z. mobilis* PDC [73] with little change in the characteristics from those of wild-type PDC (Table 3). Perhaps this is not unexpected as this substitution is found in all plant PDCs (Table 1).

A number of other side-chains interact with ThDP (Fig. 2) and it is worth noting that most are identical in *Z. mobilis* and yeast PDC. There are some conservative substitutions at zV24 (yL25), zD82 (yN83) and zD390 (yT390) and the last of these is curious because yT390 is supposed to form a hydrogen bond to one of the phosphate oxygens. Unless D390 of *Z. mobilis* PDC is protonated due to an unusually high pK_a, this hydrogen bond seems unlikely. Apart from zE50, zH113 and zH114 (see below), the role of none of the residues in close proximity to the thiamin portion of ThDP have been investigated by mutagenesis.

3. Subunit interface

3.1. General features

The four subunits of PDC are arranged as a dimer of dimers, with multiple close contacts within the dimer but rather fewer contacts between the dimers [47]. Included amongst the residues forming the interface within the dimer are zW487 (yW493) and zF496 (yF502) which are discussed further below. ThDP is wedged within the dimer interface making contact with residues from both subunits (Fig. 2). Although the three-dimensional structure of PDC in the absence of cofactors has not been determined, it is reasonable to imagine that there will be some perturbation of the dimer interface when it binds. Against this proposition is this finding that transketolase, whose structure has been determined with and without cofactors, shows very little difference in the arrangement of the subunits [103]. The major change is that two 12-residue stretches, one of which includes the C-terminal end of the ThDP-binding motif, are mobile in the apoenzyme.

3.2. Tryptophan fluorescence

Z. mobilis PDC shows quenching of tryptophan fluorescence when cofactors bind [44]. The residue responsible was identified, by mutagenesis to leucine [101], as zW487. This substitution results in an active enzyme with reduced affinity (10–15-fold) for both ThDP and Mg^{2+} (Table 3) and reduced cooperativity in the binding of these cofactors. In contrast, there is little or no effect on the ability of zW487L to bind pyruvate. The crystal structure of yeast PDC [46] shows that zW487 is far from the cofactor binding site and lies in the dimer interface close to zF496 of the adjacent subunit. Dyda et al. [46] suggested that fluorescence quenching results from perturbation of the ring stacking between zW487 and zF496 when ThDP binds. This suggested role of zF496 in fluorescence quenching led us to examine the effects of substitutions at this position [104]. Mutation to leucine, isoleucine and histidine gave enzymes that are all active and exhibit a lag phase in product formation that can be eliminated by preincubation with cofactors. All three mutants exhibit a K_m for pyruvate that is approximately twice that of the wild-type and each displays a slightly decreased affinity for Mg^{2+} and a higher affinity for ThDP (Table 3). Of greatest interest is the finding that these mutants all show the characteristic quenching of tryptophan fluorescence on addition of cofactors. Comparison of the first-order rate constant and amplitude of fluorescence quenching in these mutants with wild-type suggests [104] that there is no ring stacking between zW487 and zF496, and that zW487 must move into a more polar environment when cofactors bind.

4. Catalytic residues

4.1. Glutamate

Each of the ThDP-dependent enzymes for which the structure has been determined has a glutamate (residue 51 in yeast PDC, 59 in pyruvate oxidase and 418 in transketolase) positioned close to N-1′ of the cofactor and this has been proposed to play an essential function by promoting ionization of the C-2 proton of ThDP. This glutamate appears to be invariant across this entire class of enzymes pointing to a crucial role. Replacement of zE50, the corresponding residue in *Z. mobilis* PDC, with aspartate reduces specific activity to 3% of wild-type but we were surprised to observe that the zE50Q mutant has detectable, albeit low (0.5% of wild-type), activity [104]. The corresponding mutant of yeast PDC (yE51Q [105]) and yeast transketolase (E418Q [106]) are also active (0.04% and 2% of wild-type, respectively) as is the yeast transketolase E418A mutant (0.1% of wild-type [106]). Finding any activity when the glutamate is replaced with a neutral residue is rather unexpected given the purported role of this glutamate in the ionization of the ThDP C-2 proton. In this context, Kern et al. [107] have reported that there is no detectable ionization at C-2 of ThDP even in wild-type enzyme. They conclude that the catalytic glutamate increases the rate of proton exchange with solvent although no mechanism was proposed.

4.2. Histidines

Initially it was suggested that for yeast transketolase, C-2 ionization involved a base (H481) abstracting a proton from the 4′-amino group on the pyrimidine ring which then removed the proton from C-2 [106]. However, the finding [108] that transketolase in mammals has a glutamine at position 481 is difficult to reconcile with this hypothesis. However, in PDC there is a pair of histidines in the vicinity of the active site (Fig. 2) and these are invariant across all PDC sequences (Table 1A). Moreover, we have shown that the k_{cat}/K_m versus pH profile of *Z. mobilis* PDC is controlled by a group with a pK_a of 6.4, that might well represent a histidine, which must be protonated for the substrate to bind [109]. Consequently, it was of interest to mutate these two residues and initially each was changed to a glutamine. Mutating zH113 (yH114) results in a completely inactive protein, although it retains the ability to bind both ThDP and Mg^{2+} (Table 3). This mutant is also able to bind hydroxyethyl-ThDP and release acetaldehyde from this catalytic intermediate [109], suggesting that zH113 is mainly involved in the steps up to and including decarboxylation.

Mutating zH114 (yH115) gives an enzyme that is little different from wild-type (Table 3) apart from a 60% decrease in specific activity [109]. Recently we have shown (Huang and Duggleby, unpublished)

that zH114A is inactive and that the k_{cat}/K_m versus pH profile of zH114Q is very similar from that of wild-type; thus it appears that zH114 may play a role in orienting an intermediate in the catalytic pathway by hydrogen bonding. The role of zH113 is much more critical since zH113Q is inactive while zH113K and zH113R each shows less that 1% of wild-type activity (Huang and Duggleby, unpublished); further characterization of these last two enzymes is currently under way.

5. Substrate binding

5.1. The carboligase reaction

In all of the published structures of PDC, there appears to be little to limit the size of potential substrates as the active site is open to solvent. While little progress has been made in understanding how specificity is controlled, Bruhn et al. [110] have identified a tryptophan residue that influences the carboligase side reaction that is catalyzed by PDC.

Neuberg and Hirsch [111] were the first to show that fermenting yeast forms phenylacetylcarbinol when benzaldehyde is added to the medium. It was long suspected, but only relatively recently shown [112,113], that this carboligase reaction is catalyzed by PDC. Since it was known that yeast PDC is much more efficient at performing this reaction than *Z. mobilis* PDC [112], Bruhn et al. [110] postulated that residue 392 (W in *Z. mobilis* PDC; A in yeast PDC) was responsible for this difference and showed that the zW392A mutant is 4-fold better than wild-type in catalyzing the carboligase reaction. For a recent review of the carboligase reaction and other aspects of PDC, the reader is referred to Pohl [114].

5.2. The mobile loop

Residue 392 is rather distant from the active site (approximately 20Å from C-2 of ThDP) so the above study does not address the question of substrate specificity; rather it concerns the fate of acetaldehyde as it leaves the active site. As noted above, the active site is rather open but there are several studies [115–117] that suggest that the active site is closed during catalysis. In this context it may be relevant that residues z105–z112 (y106–z113) are invisible in the earlier PDC structures [46,47] and only partially resolved in the most recent structure [48]; these residues immediately precede the critical zH113 (yH114).

We have proposed [109] that these eight residues form a mobile loop that closes over the active site during catalysis and that substrate specificity is determined by the effectiveness of this active site closure. Moreover, since PDC from all species has a pair of branched-chain amino acids at the carboxyl end of this loop (Table 1B), it is possible that these residues may pack around the alkyl side-chain of the substrate. Recent experiments (Huang and Duggleby, unpublished) have given some support to this idea; changing zV111 (yL112) to alanine results in a small increase in the K_m for pyruvate but a larger increase in that for 2-ketovalerate. Curiously, the change in specificity is opposite to that which was predicted; it was expected that reducing the size of residue 111 would make it easier for the enzyme to accommodate larger substrates. Further mutants of zV111 (yL112) and zL112 (yL113) are currently being studied to clarify this point.

6. Envoi

Despite several decades of intensive study, several aspects of PDC catalysis and structure still tantalize us.

6.1. C-2 ionization

What is the mechanism by which ionization at C-2 of ThDP is promoted? Is there a base involved and, if so, what is its identity? Possibly it is zH113 (yH114) although Harris and Washabaugh [118] have noted the proximity of zD27 (yD28) and zE473 (yE477). Both of these residues are absolutely conserved in all PDCs and mutation of each would be informative. One mutant of each in yeast PDC has been described briefly [119]; yD28A is 7-fold less active than wild-type while yE477D shows less than 2% activity. These results suggest that the latter residue may be involved in catalysis and further mutations would be useful.

Kern et al. [107] have recently proposed that ThDP-dependent enzymes activate the cofactor by increasing the rate, rather than the extent, of C-2 ionization. This is an interesting suggestion and should be confirmed, particularly because the data appear to be inconsistent with those of Harris and Washabaugh [120]. Those workers observed that yeast PDC transferred approximately 50% of the label in C-2 tritiated ThDP to acetaldehyde but the exchange rates measured by Kern et al. [107] would suggest that all label would be lost rapidly to solvent.

6.2. Substrate specificity

What groups in the enzyme control substrate specificity? An approach to this question that has been applied successfully to many enzymes is to study the crystal structure with substrate or a competitive inhibitor bound. Regrettably, this approach is not currently feasible for PDC. Attempts to crystallize with bound substrate will inevitably fail; PDC requires only a single substrate and this will be quickly converted to products. Crystallization with acetaldehyde could be explored but this product is bound rather weakly and the high concentrations that would be needed are likely to 'pickle' the enzyme by reacting with most surface amino and sulfhydryl groups. Perhaps the enzyme-pyruvate complex could be prepared by replacing ThDP with an inactive analog such as 4'-desamino-ThDP. The possibility of using a competitive inhibitor to mimic the substrate remains beyond reach until a good inhibitor is discovered. Even close structural analogs, such as the phosphinates that are potent inhibitors of pyruvate dehydrogenase [121], have little effect on yeast [122] or *Z. mobilis* (Duggleby, unpublished) PDC.

6.3. Substrate activation

Although it is clear that residue 221 of yeast PDC is involved in activation by substrate and substrate analogs, most probably by forming a hemiketal adduct, the way in which this activates remains unclear. Determination of the structure of yeast PDC crystallized in the presence of pyruvamide [48] has not resolved this question. It is somewhat unfortunate that the mutant that has been prepared by Baburinba et al. [80] is yC221S. It could be argued that this substitution results in an enzyme that is resistant to activation, rather than permanently active. It would be instructive to replace yC221 with lysine, the corresponding residue in plant and *Z. mobilis* PDC, and with glutamate or glutamine to mimic the structures of the hemiketal formed with pyruvate and pyruvamide, respectively. Kern et al. [107] have observed that addition of pyruvamide to yeast PDC results in an increase in the rate of ThDP C-2 ionization but this observation does not clarify the molecular events by which hemiketal formation at yC221 results in a change in catalytic activity.

6.4. Smaller subunit

The existence of large (59 kDa) and small (57 kDa) subunits in PDC from yeast [5] is well established and similar observations have been made on PDC from several plants [16,20,23,25]. However, the relationship between these subunits, and their physiological function, is unclear. While the predominant view is that the smaller subunit is derived from the larger by proteolysis, this has never been demonstrated directly to our knowledge. Amino acid sequencing of the separated subunits should be a simple way of testing this hypothesis.

6.5. Mobile loop

Finally, the function of the mobile loop comprising zH105 (yQ106) to zL112 (yL113) is of interest. The mere fact that this region is mobile does not imply any functional role. However, it is clearly close to the active site since it connects zD104 (yS105) and zH113 (yH114), both of which are near the cofactor. We plan to investigate the dynamics of this loop in the near future.

Note added in proof

Recent studies on the substrate activation of yeast PDC [123,124] have clarified the role of yC221 and the mechanism by which activation is mediated. A forthcoming publication [125] will report the three-dimensional structure of *Z. mobilis* PDC.

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References

[1] C. Neuberg, L. Karczag, Biochem. Z. 36 (1911) 68–75.
[2] J. Ullrich, J.H. Wittorf, C.J. Gubler, Biochim. Biophys. Acta 113 (1966) 595–604.
[3] E. Kellermann, P.G. Seeboth, C.P. Hollenberg, Nucleic Acids Res. 14 (1986) 8963–8977.
[4] S. Hohmann, H. Cederberg, Eur. J. Biochem. 188 (1990) 615 621.
[5] B.C. Farrenkopf, F. Jordan, Protein Express. Purif. 3 (1992) 101–107.
[6] E.J. Crane III, J.A. Vaccaro, M.W. Washabaugh, J. Am. Chem. Soc. 115 (1993) 8912–8917.
[7] M.E. Alvarez, A.L. Rosa, E.D. Temporini, A. Wolstenholme, G. Panzetta, L. Patrito, H.J.F. Maccioni, Gene 130 (1993) 253–258.
[8] P. Holloway, R.E. Subden, Curr. Genet. 24 (1993) 274–277.
[9] M.M. Bianchi, L. Tizzani, M. Destruelle, L. Frontali, M. Wesolowski-Louvel, Mol. Microbiol. 19 (1996) 27–36.
[10] V. Sanchis, I. Vinas, I.N. Roberts, D.J. Jeenes, A.J. Watson, D.B. Archer, FEMS Microbiol. Lett. 117 (1994) 207–210.
[11] R.A. Lockington, G.N. Borlace, J.M. Kelly, Gene 191 (1997) 61–67.
[12] P. Holloway, R.E. Subden, Yeast 10 (1994) 1581–1589.
[13] C.S. Tsai, J.L. Shi, B.W. Beehler, B. Beck, Can. J. Microbiol. 38 (1992) 1313–1319.
[14] D.D. Davies, S. Grego, P. Kenworthy, Planta 118 (1974) 297–310.
[15] K. Wignarajah, H. Greenway, New Phytol. 77 (1976) 575–584.
[16] T.C. Lee, P.J. Langston-Unkefer, Plant Physiol. 79 (1985) 242–247.
[17] P.M. Kelley, Plant Mol. Biol. 13 (1989) 213–222.
[18] W.R. Raymond, J.B. Hostettler, K. Assar, C. Varsel, J. Food Sci. 44 (1979) 777–781.
[19] S. Leblova, J. Valik, Aust. J. Plant Physiol. 7 (1980) 35–39.
[20] U. Mücke, S. König, G. Hübner, Biol. Chem. Hoppe-Seyler 376 (1995) 111–117.
[21] U. Mücke, T. Wohlfarth, U. Fiedler, H. Bäumlein, K.P. Rücknagel, S. König, Eur. J. Biochem. 237 (1996) 373–382.
[22] K. Oba, I. Uritani, Methods Enzymol. 90 (1982) 528–532.
[23] H. Zehender, D. Trescher, J. Ullrich, Eur. J. Biochem. 167 (1987) 149–154.
[24] T.W. Kimmerer, Plant Physiol. 84 (1987) 1210–1213.
[25] J. Rivoal, B. Ricard, A. Pradet, Eur. J. Biochem. 194 (1990) 791–797.
[26] M.A. Hossain, E. Huq, A. Grover, E.S. Dennis, W.J. Peacock, T.K. Hodges, Plant Mol. Biol. 31 (1996) 761–770.
[27] E.A. Dawes, D.W. Ribbons, P.J. Large, Biochem. J. 98 (1966) 795 803.
[28] T.C. Hoppner, H.D. Doelle, Eur. J. Appl. Microbiol. Biotechnol. 17 (1983) 152–157.
[29] S. Bringer-Meyer, K.-L. Schimz, H. Sahm, Arch. Microbiol. 146 (1986) 105–110.
[30] A.D. Neale, R.K. Scopes, R.E.H. Wettenhall, N.J. Hoogenraad, J. Bacteriol. 169 (1987) 1024 1028.
[31] M.P. Stephenson, E.A. Dawes, J. Gen. Microbiol. 69 (1971) 331–343.
[32] S.E. Lowe, J.G. Zeikus, J. Gen. Microbiol. 138 (1992) 803–807.
[33] C.E. Clifton, Proc. Soc. Exp. Biol. Med. 43 (1940) 585–587.
[34] J. De Ley, J. Schell, J. Gen. Microbiol. 29 (1962) 589–601.
[35] A. Haq, E.A. Dawes, J. Gen. Microbiol. 68 (1971) 295–306.
[36] J. Swings, J. De Ley, Bacteriol. Rev. 41 (1977) 1 46.
[37] M. Gibbs, R.D. DeMoss, J. Biol. Chem. 207 (1954) 689–694.
[38] T. Conway, FEMS Microbiol. Rev. 103 (1992) 1–28.
[39] H. An, R.K. Scopes, M. Rodriguez, K.F. Keshav, L.O. Ingram, J. Bacteriol. 173 (1991) 5975–5982.
[40] A.D. Neale, R.K. Scopes, R.E.H. Wettenhall, N.J. Hoogenraad, Nucleic Acids Res. 15 (1987) 1753–1761.
[41] P. Kenworthy, D.D. Davies, Phytochemistry 15 (1976) 279–282.
[42] T.P. Singer, J. Pensky, J. Biol. Chem. 196 (1952) 375–388.
[43] S. König, D. Svergun, M.H.J. Koch, G. Hübner, A. Schellenberger, Biochemistry 31 (1992) 8726 8731.
[44] R.J. Diefenbach, R.G. Duggleby, Biochem. J. 276 (1991) 439–445.
[45] M. Pohl, J. Grötzinger, A. Wollmer, M.-R. Kula, Eur. J. Biochem. 224 (1994) 651–661.
[46] F. Dyda, W. Furey, S. Swaminathan, M. Sax, B. Farrenkopf, F. Jordan, Biochemistry 32 (1993) 6165–6170.
[47] P. Arjunan, T. Umland, F. Dyda, S. Swaminathan, W. Furey, M. Sax, B. Farrenkopf, Y. Gao, D. Zhang, F. Jordan, J. Mol. Biol. 256 (1996) 590–600.
[48] G. Lu, D. Dobritzsch, S. König, G. Schneider, FEBS Lett. 403 (1997) 249–253.
[49] G. Miczka, M.-R. Kula, Anal. Lett. 22 (1989) 2771–2782.
[50] R.F.W. Hopmann, Eur. J. Biochem. 110 (1980) 311–318.
[51] J. Ullrich, H. Freisler, Hoppe-Seyler's Z. Physiol. Chem. 358 (1977) 318.
[52] M. Sieber, S. König, G. Hübner, A. Schellenberger, Biomed. Biochim. Acta 42 (1983) 343–349.
[53] J. Ullrich, H. Zehender, in A. Schellenberger, R.L. Schowen (Eds.), Thiamin Pyrophosphate Biochemistry, Vol. 1, CRC Press, Boca Raton, FL, 1988, pp. 75–78.
[54] H.D. Schmitt, F.K. Zimmermann, J. Bacteriol. 151 (1982) 1146–1152.
[55] S. Hohmann, J. Bacteriol. 173 (1991) 7963–7969.
[56] J.M. Candy, R.G. Duggleby, J.S. Mattick, J. Gen. Microbiol. 137 (1991) 2811 2815.
[57] M. Killenberg-Jabs, S. König, S. Hohmann, G. Hübner, Biol. Chem. Hoppe-Seyler 377 (1996) 313–317.

[58] J. Ullrich, J. Kellermann, W. Schmidt, in H. Bisswanger, J. Ullrich (Eds.), Biochemistry and Physiology of Thiamin Diphosphate Enzymes, VCH, Weinheim, 1990, pp. 99–105.
[59] H. Zehender, J. Ullrich, FEBS Lett. 180 (1985) 51–54.
[60] V.M. Peschke, M.M. Sachs, Mol. Gen. Genet. 240 (1993) 206–212.
[61] A.L. Rosa, M.E. Alvarez, D. Lawson, H.J.F. Maccioni, Biochem. J. 268 (1990) 649–655.
[62] T.L. Bowen, J. Union, D.L. Tumbula, W.B. Whitman, Gene 188 (1997) 77–84.
[63] A. Schellenberger, G. Hübner, Hoppe-Seyler's Z. Physiol. Chem. 348 (1967) 491–500.
[64] G. Hübner, S. König, A. Schellenberger, M.H.J. Koch, FEBS Lett. 266 (1990) 17–20.
[65] D.E. Green, D. Herbert, V. Subrahmanyan, J. Biol. Chem. 38 (1941) 327–339.
[66] L.O. Krampitz, in L.O. Krampitz (Ed.), Thiamin Diphosphate and its Catalytic Functions, Marcel Decker, New York, 1970, pp. 4–26.
[67] A.D. Gounaris, I. Turkenkopf, L.L. Civerchia, J. Greenlie, Biochim. Biophys. Acta 405 (1975) 492–499.
[68] S. Eppendorfer, S. König, R. Golbik, H. Neef, K. Lehle, R. Jaenicke, A. Schellenberger, G. Hübner, Biol. Chem. Hoppe-Seyler 374 (1993) 1129–1134.
[69] A. Schellenberger, Angew. Chem. Int. Edn. 6 (1967) 1024–1035.
[70] A. Schellenberger, Ann. NY Acad. Sci. 378 (1982) 51–62.
[71] G. Hübner, in A. Schellenberger, R.L. Schowen (Eds.), Thiamin Pyrophosphate Biochemistry, Vol. 1, CRC Press, Boca Raton, FL, 1988, pp. 93–100.
[72] R. Golbik, H. Neef, G. Hübner, S. König, B. Seliger, L. Meshalkina, G.A. Kochetov, A. Schellenberger, Bioorg. Chem. 19 (1991) 10–17.
[73] J.M. Candy, R.G. Duggleby, Biochem. J. 300 (1994) 7–13.
[74] J. Ullrich, I. Donner, Hoppe-Seyler's Z. Physiol. Chem. 351 (1970) 1026–1029.
[75] G. Hübner, R. Weidhase, A. Schellenberger, Eur. J. Biochem. 92 (1978) 175–181.
[76] G. Hübner, A. Schellenberger, Biochem. Int. 13 (1986) 767–772.
[77] J. Ullrich, Ann. NY Acad. Sci. 378 (1982) 287–305.
[78] A. Schellenberger, G. Hübner, M. Sieber, in A. Schellenberger, R.L. Schowen (Eds.), Thiamin Pyrophosphate Biochemistry, Vol. 1, CRC Press, Boca Raton, FL, 1988, pp. 113–121.
[79] S. König, D. Svergun, M.H.J. Koch, G. Hübner, A. Schellenberger, Eur. Biophys. J. 22 (1993) 185–194.
[80] I. Baburina, Y. Gao, Z. Hu, F. Jordan, S. Hohmann, W. Furey, Biochemistry 33 (1994) 5630–5635.
[81] S. Sun, R.G. Duggleby, R.L. Schowen, J. Am. Chem. Soc. 117 (1995) 7317–7322.
[82] S. Sun, G.S. Smith, M.H. O'Leary, R.L. Schowen, J. Am. Chem. Soc. 119 (1997) 1507–1515.
[83] S. König, in A. Schellenberger, R.L. Schowen (Eds.), Thiamin Pyrophosphate Biochemistry, Vol. 1, CRC Press, Boca Raton, FL, 1988, pp. 123–130.
[84] H. Lehmann, G. Fischer, G. Hübner, K.-D. Kohnert, A. Schellenberger, Eur. J. Biochem. 32 (1973) 83–87.
[85] H. Uhlemann, A. Schellenberger, FEBS Lett. 63 (1976) 37–39.
[86] G. Gish, T. Smyth, R. Kluger, J. Am. Chem. Soc. 110 (1988) 6230–6234.
[87] G. Thomas, R. Diefenbach, R.G. Duggleby, Biochem. J. 266 (1990) 305–308.
[88] A. Boiteux, B. Hess, FEBS Lett. 9 (1970) 293–296.
[89] H. van Urk, D. Schipper, G.J. Breedveld, P.R. Mak, W.A. Scheffers, J.P. van Dijken, Biochim. Biophys. Acta 992 (1989) 78–86.
[90] J.M. Candy, Ph.D. Thesis, Biochemistry Department, University of Queensland, 1993.
[91] R. Breslow, Ann. NY Acad. Sci. 98 (1962) 445–452.
[92] R. Kluger, Chem. Rev. 87 (1987) 863–876.
[93] J.H. Wittorf, C.J. Gubler, Eur. J. Biochem. 14 (1970) 53–60.
[94] J. Ullrich, I. Donner, Hoppe-Seyler's Z. Physiol. Chem. 351 (1970) 1030–1034.
[95] J. Ullrich, A. Wollmer, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 1635–1644.
[96] F. Jordan, O. Akinyosoye, G. Dikdan, A. Kudzin, D. Kuo, in A. Schellenberger, R.L. Schowen (Eds.), Thiamin Pyrophosphate Biochemistry, Vol. 1, CRC Press, Boca Raton, FL, 1988, pp. 79–92.
[97] C.F. Hawkins, A. Borges, R.N. Perham, FEBS Lett. 255 (1989) 77–82.
[98] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673–4680.
[99] Y. Lindqvist, G. Schneider, U. Ermler, M. Sundström, EMBO J. 11 (1992) 2373–2379.
[100] Y.A. Muller, G.E. Schultz, Science 259 (1993) 965–967.
[101] R.J. Diefenbach, J.M. Candy, J.S. Mattick, R.G. Duggleby, FEBS Lett. 296 (1992) 95–98.
[102] G.C. Russell, R.S. Machado, J.R. Guest, Biochem. J. 287 (1992) 611–619.
[103] M. Sundström, Y. Lindqvist, G. Schneider, FEBS Lett. 313 (1992) 229–231.
[104] J.M. Candy, J. Koga, P.F. Nixon, R.G. Duggleby, Biochem. J. 315 (1996) 745–751.
[105] M. Killenberg-Jabs, S. König, I. Eberhardt, S. Hohmann, G. Hübner, Biochemistry 36 (1997) 1900–1905.
[106] C. Wikner, L. Meshalkina, U. Nilsson, M. Nikkola, Y. Lindqvist, M. Sundström, G. Schneider, J. Biol. Chem. 269 (1994) 32144–32150.
[107] D. Kern, G. Kern, H. Neef, K. Tittman, M. Killenberg-Jabs, C. Wikner, G. Schneider, G. Hübner, Science 275 (1997) 67–70.
[108] G. Schenk, R. Layfield, J.M. Candy, R.G. Duggleby, P.F. Nixon, J. Mol. Evol. 44 (1997) 552–572.
[109] G. Schenk, F.J. Leeper, R. England, P.F. Nixon, R.G. Duggleby, Eur. J. Biochem. 248 (1997) 73–81.
[110] H. Bruhn, M. Pohl, J. Grötzinger, M.-R. Kula, Eur. J. Biochem. 234 (1995) 650–655.
[111] C. Neuberg, J. Hirsch, Biochem. Z. 115 (1921) 282–310.

[112] S. Bringer-Meyer, H. Sahm, Biocatalysis 1 (1988) 321–331.
[113] D.H.G. Crout, H. Dalton, D.W. Hutchinson, M. Miyagoshi, J. Chem. Soc. Perkin Trans. 1 (1991) 1329–1334.
[114] M. Pohl, Adv. Biochem. Eng. Biotechnol. 58 (1997) 15–43.
[115] R. Kluger, T. Smyth, J. Am. Chem. Soc. 103 (1981) 1214–1216.
[116] F.J. Alvarez, J. Ermer, G. Hübner, A. Schellenberger, R.L. Schowen, J. Am. Chem. Soc. 117 (1995) 1678–1683.
[117] M. Lobell, D.H.G. Crout, J. Chem. Soc. Perkin Trans. 1 (1996) 1577–1581.
[118] T.K. Harris, M.W. Washabaugh, Biochemistry 34 (1995) 14001–14011.
[119] F. Jordan, I. Baburina, Y. Gao, F. Guo, A. Kahyaoglu, N. Nemeria, A. Volkov, J.-Z. Yi, D. Zhang, R. Machado, J.R. Guest, W. Furey, S. Hohmann, in H. Bisswanger, A. Schellenberger (Eds.) Biochemistry and Physiology of Thiamin Diphosphate Enzymes, A. u. C. Intemann Wissenschaftlicher Verlag, Prien, 1996, pp. 53–69.
[120] T.K. Harris, M.W. Washabaugh, Biochemistry 34 (1995) 13994–14000.
[121] B. Laber, N. Amrhein, Biochem. J. 248 (1987) 351–358.
[122] M. Spinka, G. Hübner, in H. Bisswanger, A. Schellenberger (Eds.), Biochemistry and Physiology of Thiamin Diphosphate Enzymes, A. u. C. Intemann Wissenschaftlicher Verlag, Prien, 1996, pp. 186–194.
[123] I. Baburina, H. Li, B. Bennion, W. Furey, F. Jordan, Biochemistry 37 (1998) 1235–1244.
[124] I. Baburina, G. Dikdan, F. Guo, G.I. Tous, B. Root, F. Jordan, Biochemistry 37 (1998) 1245–1255.
[125] D. Dobritzsch, S. König, G. Schneider, G. Lu, J. Biol. Chem. (1998) in press.